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Marine hydrocarbonoclastic bacteria

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Abstract: Several marine bacteria that specialize in the degradation of hydrocarbons have been isolated from polluted seawater around the world. Some of these bacteria can use exclusively hydrocarbons as growth substrates and are known as 'obligate hydrocarbonoclastic bacteria' (OHCB). Marine hydrocarbon-degrading bacteria are not only powerful tools for bioremediation but also an extraordinary archive of mono and dioxygenases, oxidases, dehydrogenases and other enzymatic activities with several applications in regio- and stereo-selective biosynthesis. Furthermore, marine hydrocarbon-degrading microorganisms often synthesize very peculiar compounds related to their adaptation to grow in hydrocarbon-rich environments, such as bio-detergents with high emulsifying activity and polymeric storage substances of industrial interest. This chapter describes in detail the features and the most interesting products of this subset of hydrocarbon-degrading bacteria.

Key words: hydrocarbon, oxygenase, oxidase, biosurfactants, storage lipid.

14.1 Introduction

Hydrocarbons are the most widespread pollutants. Both natural and anthropogenic sources contribute to diffuse aromatic and aliphatic

hydrocarbons into almost every environment, from urban soils of temperate regions to the ice of poles.

Specialized microbial metabolisms constantly produce both simple aliphatic hydrocarbons, like methane, ethane, propane, butane, ethylene, and complex high molecular weight hydrocarbons like pristane and phytane (Ladygina et al. 2006; Taylor et al. 2000). Plants produce a large variety of hydrocarbons too (Fuentes et al. 2000). High molecular weight isoprenoid hydrocarbons are major constituents of the cuticle of insects (Golebiowski et al. 2011) and several polycyclic aromatic hydrocarbons (PAHs) are produced by forest fires (Vergnoux et al. 2011).

The most important anthropogenic sources of hydrocarbons include combustion in engines and incinerators, direct release from industry, industrial wastes and, obviously, fossil fuel spills. Crude oil and all fuels derived from oil distillation are among the main sources of hydrocarbons released into the environment. Spills take place not only during accidents involving oil tankers, such as collisions and groundings, but result from routine daily operations, among others extraction from terrestrial and submarine oil wells, loading and discharging cargo, and tanking.

Crude oil and fuels contain saturated, unsaturated and aromatic hydrocarbons (Henry 1998; King 1988). These fractions are extremely complex including hundreds of compounds. For example, the aromatic fraction, which may constitute from about 10% to more than 40% of total hydrocarbons, contains (poly)alkyl-benzenes, PAHs, (poly)alkyl-PAHs, naphthenes (i.e. polycyclic compounds with fused aromatic and saturated rings) and heterocyclic compounds.

Most of the hydrocarbons are quite stable and accumulate into the environment. Moreover, they are a concentrated source of energy and carbon. Thus, it is not surprising that an impressive number of microorganisms have evolved the ability to use them as substrates for growth (Rosenberg 1992; Van Hamme et al. 2003 and references therein). This ability is widely distributed and can be found in almost every group of microorganisms including archea, bacteria and fungi, mesophiles and extremophiles. Interestingly, several microorganisms are able to use one or more hydrocarbons as the sole source of carbon and energy and some strains are so specialized that they can use exclusively hydrocarbons. This surprising feature is especially diffused among marine bacteria as discussed in section 14.2.

The chemical stability and the diversity of hydrocarbons have induced microorganisms to develop a large variety of interesting and, often, unique enzymatic activities involved in the activation and fragmentation of hydrocarbons to smaller molecules which can enter the central

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energetic and biosynthetic metabolism. Usually, in the aerobic metabolism, activation of hydrocarbons is mediated by the addition of one or two oxygen atoms which provide reactive molecules for subsequent reactions. For example, the metabolism of methane and linear alkanes starts with a terminal monooxygenation reaction providing a primary alcohol which can be further oxidized to carboxylic acid (Rojo 2009; van Beilen and Funhoff 2007) (Figure 14.1a, b).

Alkenes, like ethylene and styrene, are oxygenated to epoxides which are further metabolized through several different routes (Mooney et al. 2006; van Beilen and Funhoff 2007; van Hylckama Vlieg and Janssen 2001) (Figure 14.1c). Cyclic alkanes are hydoxylated to secondary alcohols, oxidized to ketones and further oxygenated to give esters which finally undergo hydrolysis (Figure 14.1d). The metabolism of branched alkanes and especially of aromatic hydrocarbons is much more complex (Seo et al. 2009). Figure 14.2 shows the variety of known degradative pathways of

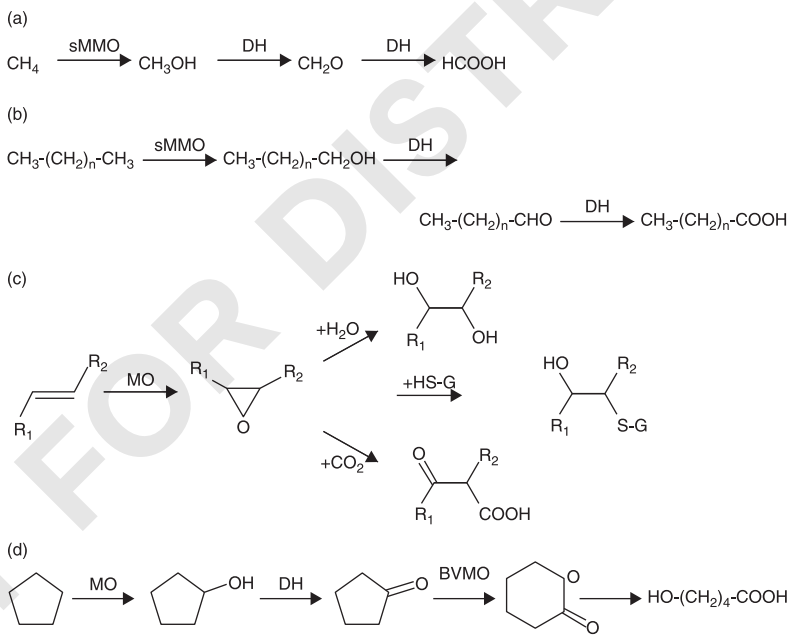

Figure 14.1 **Common degradative pathways of aliphatic hydrocarbons: (a) methane, (b) linear alkanes, (c) alkenes, (d) cyclopentane. sMMO, soluble methane monooxygenase; MO, monooxygenase; DH, dehydrogenase; BVMO, Baeyer-Villiger monooxygenase; HS-G, glutathione**

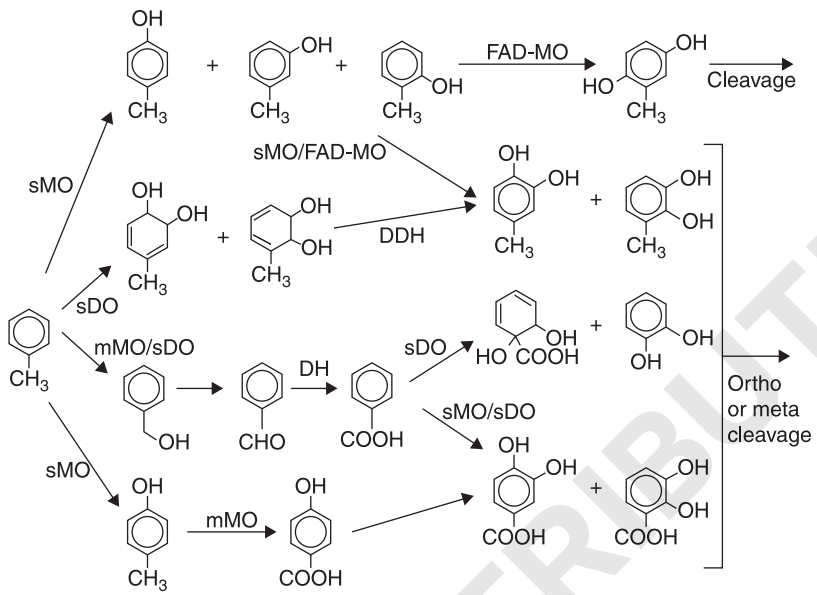

Figure 14.2 **Possible upper patways for toluene degradation. sMO, soluble monooxygenase; mMO, membrane monooxygenase; FAD-MO, FAD-dependent monooxygenase; sDO, soluble dioxygenase; DDH, dihydrodiol dehydrogenase; DH, dehydrogenase**

toluene (Jindrova et al. 2002), a quite simple monocyclic aromatic hydrocarbon. Initial oxidation can occur on the methyl group thus providing benzoic acid or on the ring producing methyl-catechols or methyl-hydroquinone. The two hydroxyl groups of methyl-catechol can be added by two sequential hydroxylation reactions or by a dioxygenation reaction. Also the cleavage of the aromatic ring of catechols can be obtained in two different ways –the extradiol and intradiol cleavages (Figure 14.3) – each starting a different 'lower pathway' which provides intermediates for the central metabolism. More possibilities may be discovered in the future.

The degradation of PAHs is similar to that of monocyclic ones even if usually the rings are sequentially hydroxylated and cleaved (Seo et al. 2009) as shown for (methyl)naphthalene in Figure 14.4. The metabolism of nitrogen and oxygen containing heterocyclic aromatic compounds starts either by lateral or angular dioxygenation (Figure 14.5a) (Seo et al. 2009), whereas, quinoline and isoquinoline are usually monohydroxylated at the pyridine ring (Figure 14.5b). Metabolism of sulfur containing heterocyclic aromatic compounds such as dibenzothiophene starts

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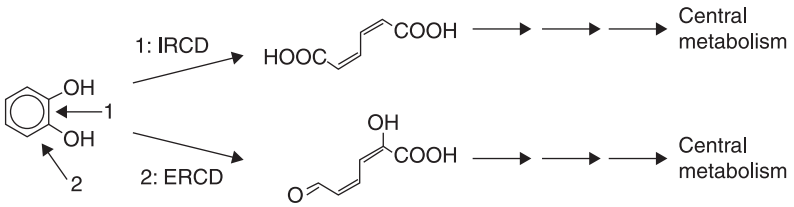

Figure 14.3 Possible lower patways for degradation of catechols. ERCD, extradiol ring cleavage dioxygenase (meta cleavage); IRCD, intradiol ring cleavage dioxygenase (ortho cleavage)

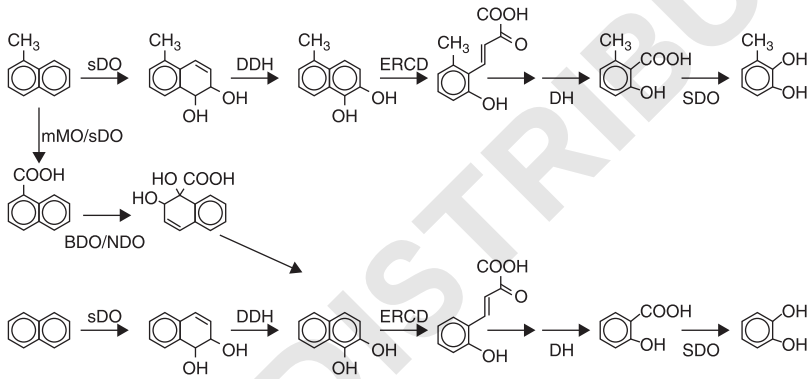

Figure 14.4 Degradative pathways of PAHs. sMO, soluble monooxygenase; mMO, membrane monooxygenase; FAD-MO, FAD-dependent monooxygenase; sDO, soluble dioxygenase; DDH, dihydrodiol dehydrogenase; DH, dehydrogenase; ERCD, extradiol ring cleavage dioxygenase; SDO, salicylate dioxygenase; BDO/NDO benzoate, naphtoate dioxygenase

either by oxidation of the ring (Figure 14.5a) or of the sulfur atom (Seo et al. 2009).

All the mono (MO) and dioxygenases (DO) involved in these complex pathways are very interesting enzymes both for their peculiar chemistry and as potential tools for biosynthesis. A noteworthy example are soluble methane MOs from bacteria which grow using methane as the sole carbon and energy source (Tinberg and Lippard 2011). These soluble cytosolic multicomponent enzymes include a reductase component which oxidizes NAD(P)H providing two electrons to an oxygenase complex with structure $(\alpha\beta\gamma)_2B_2$.

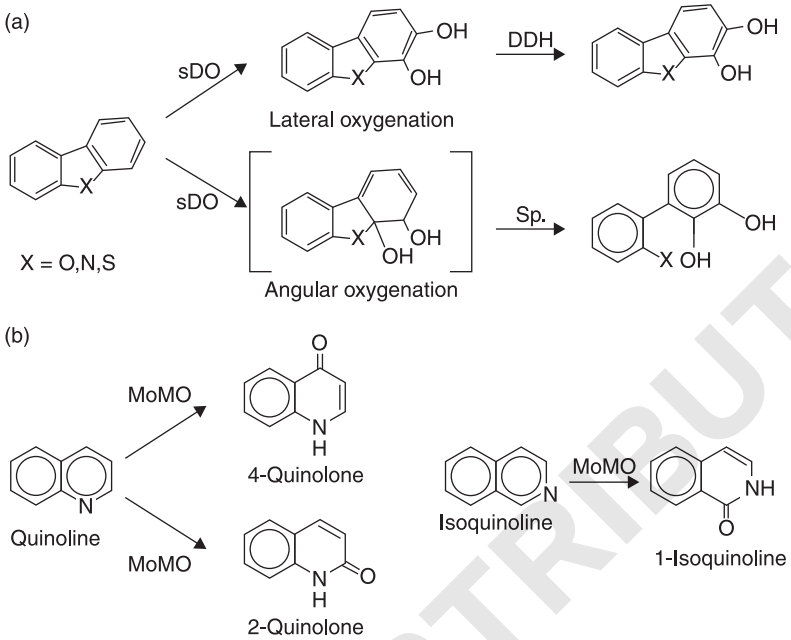

Figure 14.5 **Degradative pathways eterocyclic aromatic compounds (a) and quinolines (b). sMO, soluble monooxygenase; DDH, dihydrodiol dehydrogenase; Sp., spontaneous transformation; MoMO, molybdopteryn dependent monooxygenase**

The B subunit acts as a regulatory component controlling the electron flux and the accessibility of the active site located in the subunit α. The active site contains two non heme iron (III) ions bridged by a hydroxyl anion. The electrons provided by the reductase reduce the Fe(III)-OH-Fe(III) cluster to Fe(II)-OH(H)-Fe(II) which in turn reacts with dioxygen generating a high-valence diiron cluster whose oxidizing power is high enough to insert an oxygen atom into the C-H bond of methane. The details of the mechanism are still under debate; however, these studies have already allowed to develop synthetic mimetics useful as catalysts in the chemical industry (Do LH 2011).

Methane MOs belong to the so-called bacterial multicomponent MOs (BMMs) which include several families of enzymes with a very similar mechanism but different substrate specificity (Notomista et al. 2003) like alkene epoxidases, benzene/toluene/xylene MOs (BTX-MOs) and phenol hydroxylases (PHs). BTX-MOs and PHs are specialized in the regioselective monooxygenation of aromatic rings, a reaction very

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difficult to reproduce with conventional catalysts which usually provides complex mixtures of products, including polymeric byproducts, thus lowering the yield and making the purification difficult. Therefore it is not surprising that several research lines try to exploit these enzymes to develop biosynthetic processes (Nolan and O'Connor 2008; Notomista et al. 2011).

Membrane MOs are an unrelated family of non heme iron dependent-MOs which are specialized in the monooxygenation of aliphatic (AlkB-like alkane monooxygenases) or aromatic methyl groups of mono and polycyclic hydrocarbons (XylM-like monooxygenases) (Austin et al. 2003; van Beilen and Funhoff 2007). XylM-like monooxygenases produce benzyl-alcohols and benzaldehydes which are converted to benzoic acids, naphthoic acids etc. by dehydrogenases (Figure 14.2 and 14.4). The characterization and the exploitation of these MOs has been hampered by the fact that they are bound to the membrane.

In quinolines and isoquinolines the heterocyclic nitrogen atom deactivate the pyridine ring; thus the initial hydroxylation usually is performed by peculiar MOs which use a molybdenum containing cofactor (molybdopteryn) as redox center (Bonin et al. 2004; Hille 2005). These molybdopteryn-dependent oxygenases (MoMOs) can catalyze other difficult reactions such as the monooxygenation of xantine and the oxidation of carbon monoxide to CO_2 (Hille 2005).

On the contrary, phenolic hydroxyl groups increase the reactivity of an aromatic ring; indeed, some phenol hydroxylases use FAD or FMN to activate dioxygen instead of metals (Ballou et al. 2005). Some of these FAD/FMN-PHs hydroxylate position in para to a preexisting hydroxyl group thus producing hydroquinone instead of catechol, the product of non-heme iron dependent-PHs.

Another unrelated family of MOs is that of Baeyer-Villiger MOs which catalyze the conversion of ketones to esters (Leisch et al. 2011). They are involved in the metabolism of cyclic alkanes (Figure 14.1) and of some naphthenes (Seo et al. 2009). As the monooxygenation of ketones is a very useful reaction for the chemical industry, BVMOs are considered very valuable tools to develop biocatalytic processes (Leisch et al. 2011).

Soluble hydroxylating dioxygenases are similar to BMM for several aspects. They are complex multicomponent enzymes which include a reductase which oxidizes NADH and provides electron to a hydroxylase complex (Ferraro et al. 2005). The hydroxylase contains Fe(III) as co-factor, however, a single iron ion is bound into the active site. Therefore the two electrons are transferred sequentially and dioxygen is activated to a reactive species which transfers both atoms to the aromatic ring thus

producing dihydrodiols (Figure 14.2). sDO are capable of catalyzing also the hydroxylation of aromatic methyl groups thus mimicking membrane MOs. Like BTX-MOs and PHs, sDO are very interesting enzymatic tools for biosynthesis both alone, to prepare dihydrodiols which are useful synthons for chemical synthesis, and coupled to dehydrogenases, to produce dihydroxylated aromatic molecules (Boyd and Bugg 2006; Nolan and O'Connor 2008).

Extra and intradiol ring cleavage DOs (ERCD and IRCD) are simpler soluble enzymes composed by one or two subunits (Vaillancourt et al. 2006). Both use iron as cofactor and catechols bind to the iron ion as bidentate ligands. The main differences between the two types of DOs are that ERCDs contain a Fe(II) ion and catechol binds as a monoanion, whereas IRCDs contain Fe(III) and catechol binds as a dianion (Vaillancourt et al. 2006). These differences determine the different regioselectivity. Thus far their biosynthetic potential has not been exploited.

With the exception of RCDs and MoMOs all the oxygenases described above require a source of electrons – usually NAD(P)H. This makes their use in industrial processes more complex and has stimulated the development of intriguing and imaginative solutions. In some cases whole cells expressing the recombinant enzyme are used as catalyst (Notomista et al. 2011). In other cases the oxygenase has been coupled to a second enzyme which, oxidizing a cheap substrate like formic acid or phosphite, regenerates the reduced coenzyme (Leisch et al. 2011). Some BVMOs have been successfully prepared as fusion proteins with a 'recycling enzyme' (Leisch et al. 2011).

Hydrocarbon degrading microorganisms are a source of several other very interesting products related to their adaptation to grow in hydrocarbon rich environments, such as bio-detergents with very high emulsifying activity and polymeric storage substances (Rosenberg 1992; Van Hamme et al. 2003 and references therein).

Marine hydrocarbon-degrading bacteria are a particularly rich source of enzymes and non-enzymatic bio-molecules of industrial interest. The next sections describe in detail the features and the most interesting products of this subset of hydrocarbon-degrading bacteria.

14.2 Marine hydrocarbonoclastic bacteria

The aerobic degradation of hydrocarbons by marine microorganisms has been extensively studied in the last decades, and oil-degrading marine bacteria have been isolated from different sites around the world (Head

et al. 2006) showing the widespread diffusion of these bacteria in the environment. Hydrocarbon-degrading microorganisms usually exist in very low abundance in marine environments. Pollution by petroleum hydrocarbons, however, may stimulate the growth of such organisms and cause changes in the structure and composition of microbial communities in the contaminated area.

Identification of the key organisms that play roles in the biodegradation of pollutants is important for understanding, evaluating and developing *in situ* bioremediation strategies. Furthermore, due to the increasing biotechnological applications of enzymes from hydrocarbon degrading bacteria, many efforts have been made to characterize bacterial communities, to identify possible degraders, and to elucidate the catalytic potential of their enzymes.

In contrast to terrestrial hydrocarbon degraders which utilize a large range of organic substrates, their marine counterparts are mostly highly specialized hydrocarbon utilizers. They constitute the group of 'marine hydrocarbonoclastic bacteria' (Yakimov et al. 2007). Most of the members of this group show a remarkably narrow substrate spectrum limited almost exclusively to hydrocarbons (alkanes or polycyclic aromatic hydrocarbons) and a few organic acids, and are thus named 'obligate hydrocarbonoclastic bacteria' (OHCB). They are mainly bacteria of the genera *Alcanivorax* (Yakimov et al. 1998), *Cycloclasticus* (Dyksterhouse et al. 1995), *Marinobacter* (Gauthier et al. 1992), *Neptunomonas* (Hedlund et al. 1999), *Oceanobacter* (Teramoto et al. 2009), *Oleiphilus* (Golyshin et al. 2002), *Oleispira* (Yakimov et al. 2003) and *Thalassolituus* (Yakimov et al. 2004).

OHCB can be divided into two groups on the basis of whether they degrade aliphatic or aromatic hydrocarbons. *Alcanivorax, Marinobacter, Oleiphilus, Oleispira, Oceanobacter* and *Thalassolituus* spp. use a variety of branched- and/or straight-chain saturated hydrocarbons (Wentzel et al. 2007), whereas *Neptunomonas* and *Cycloclasticus* spp. have evolved to use a range of polycyclic aromatic hydrocarbons. However, many other 'non-obligate' hydrocarbonoclastic bacteria of the genera, *Vibrio, Pseudoalteromonas, Marinomonas* and *Halomonas* have been isolated as marine bacteria capable of degrading naphthalene, phenanthrene and chrysene (Melcher et al. 2002).

Genera *Alcanivorax* and *Cycloclasticus* include both OHCB, such as *A. borkumensis, A. jadensis* (Fernandez-Martinez et al. 2003; Golyshin et al. 2002), *A. dieselolei* (Liu and Shao 2005), *C. pugetii* (Dyksterhouse et al. 1995) and *C. oligotrophus* (Robertson et al. 1998), and more nutritionally versatile species with less restricted substrate range, like *A.*

venustensis and *C. spirillensus* (Chung and King 2001; Fernandez-Martinez et al. 2003).

Oleiphilus messinensis (Golyshin et al. 2002), *Oleispira antarctica* (Yakimov et al. 2003), *Thalassolituus oleivorans* (Yakimov et al. 2004) and *Neptunomonas naphthovorans* (Hedlund et al. 1999) are the most representative members of their OHCB groups.

Several studies have shown that an influx of oil in a marine site causes population densities of OHCB to transiently increase up to 90% of the total microbial community. *Alcanivorax* and *Cycloclasticus* spp. have been shown to be rapidly and strongly selected after hydrocarbon pollution, being the two key organisms with major roles in the degradation of aliphatic and aromatic hydrocarbons, respectively (Golyshin et al. 2005; Harayama et al. 1999, 2004; Head et al. 2006).

Alcanivorax spp., for example, were shown to increase from being undetectable in pristine seawater to constituting 70–90% of prokaryotic cells in oil-polluted seawater within 1–2 weeks (Harayama et al. 1999; Kasai et al. 2002a; Syutsubo et al. 2001). It has been suggested that the success of *Alcanivorax* spp. could be related to their ability to use branched-chain alkanes more effectively than do other hydrocarbon-degrading bacteria, giving these species a selective advantage (Hara et al. 2003). After an initial rapid increase in population size, *Alcanivorax* spp. decline to much lower numbers within a few weeks. This phenomenon is related to the depletion of saturated hydrocarbons, suggesting a specialized character of *Alcanivorax* strains to degrade these compounds (Head et al. 2006).

There is an analogous situation for *Cycloclasticus* spp., which were found to be particularly enriched in oil-contaminated seawater (Head et al. 2006; Kasai et al. 2002b; McKew et al. 2007; Niepceron et al. 2010) and seem to have a global role in the removal of aromatic hydrocarbons (Kasai et al. 2002b). Noteworthy, the predominance of *Alcanivorax* and *Cycloclasticus* spp. in early stages of petroleum degradation has also been reported in microcosms and mesocosms studies (Cappello et al. 2007; Roling et al. 2002).

14.3 Bioactive compounds from hydrocarbon-degrading microorganisms

Ever since the capacity of marine hydrocarbon-degrading microorganisms to efficiently degrade hydrocarbons and their potential use for the

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mitigation of oil spills was assessed, it has become apparent that such bacteria might also be an answer to the increasing needs of the biotechnology-based economy (Yakimov et al. 2007) for the production of bioactive compounds (section 14.3.2) or as a source of novel enzymatic activities (section 14.4).

14.3.1 Biosurfactants

The interest in microbial biosurfactants has steadily increased during the past decade. Biosurfactants have several advantages over chemical surfactants including lower toxicity, environmental compatibility and higher biodegradability (Lima et al. 2011; Zajic et al. 1977). Commercialization of biosurfactants (Banat et al. 2000; Desai and Banat 1997) in the cosmetic, food, health care, pulp- and paper-processing, coal, ceramic, and metal industries has been proposed. They have also been found to possess several properties of therapeutic and biomedical importance: they have antibacterial, antifungal and antiviral properties and anti-adhesive action against several pathogenic microorganisms (Cameotra and Makkar 2004; Rodrigues et al. 2006; Singh and Cameotra 2004). Furthermore, the most promising applications are the clean-up of oil-contaminated tankers, recovery of crude oil and bioremediation of sites contaminated with hydrocarbons, heavy metals, and other pollutants (Mulligan 2005; Slizovskiy et al. 2011).

Why should marine hydrocarbonoclastic bacteria be a valuable source of novel biosurfactants? Bacteria able to grow on petroleum-derived hydrocarbons have developed several strategies (McGenity et al. 2012; Wentzel et al. 2007) for the uptake of these hydrophobic compounds characterized, among others, by very low bioavailability. Cell-surface hydrophobicity and biosurfactants production (Bertrand et al. 1993; Desai and Banat 1997; Satpute et al. 2010; Weiner 1997) play a major role in regulating the adhesion and detachment of microorganisms to the growth substrates (Ron and Rosenberg 2001). Both aspects can improve the bioavailability of hydrocarbons to the microbial cells by enhancing the formation of oil-water emulsions and by increasing the contact area between bacteria and water-insoluble hydrocarbons (Ron and Rosenberg 2001, 2002). This increases the rate of hydrocarbons dissolution and their use by microorganisms.

Microbial biosurfactants are amphiphilic extracellular compounds containing both a hydrophilic and a hydrophobic moiety and therefore are capable of reducing surface tension and facilitate hydrocarbon uptake

and emulsification/dispersion (Ron and Rosenberg 2001). They are generally grouped as low or high molecular weight biosurfactants (Rosenberg and Ron 1999). Both low and high molecular weight biosurfactants contain lipid moieties attached to a variety of molecules forming glycolipids, lipoproteins, lipopolysaccharides, etc. (Ron and Rosenberg 2001, 2002). Low molecular weight molecules efficiently lower surface and interfacial tensions whereas high molecular weight polymers bind tightly to surfaces (Ron and Rosenberg 2002).

It is worth underlining that large-scale production of these molecules has not been achieved yet because of low yields in production processes and high recovery and purification costs. Thus, to reduce the production cost it is desirable to use low-cost raw materials (Makkar and Cameotra 2002; Mukherjee et al. 2006). A variety of cheap raw materials, including plant-derived oils and oil wastes, have been reported to support biosurfactants production; in this context hydrocarbonoclastic bacteria constitute a unique opportunity to couple bioremediation and biotechnological production.

Low molecular weight biosurfactants

Low molecular weight biosurfactants are generally glycolipids in which carbohydrates are attached to a long-chain aliphatic acid, or lipopeptides. Glycolipid bioemulsifiers, such as rhamnolipids, trehalose lipids and sophorolipids, are disaccharides that are acylated with long-chain fatty acids or hydroxy fatty acids.

Alcanivorax strains represent one of the most studied group of n-alkane degraders able to produce biosurfactants. Recently, a new class of glycolipids, glucose lipids, produced by *Alcanivorax borkumensis* SK2 (Yakimov et al. 1998), has been described (Abraham et al. 1998). The strain uses aliphatic hydrocarbons as its main carbon source for growth and produces an anionic glucose lipid biosurfactant. This glucose-containing compound possesses β-anomeric glucose connected to tetrameric β-hydroxy-decanoic acid. The four β-hydroxy fatty acids are linked together by ester bonds and coupled glycosidically with C1 of glucose. Golyshin et al. (2003) reported that cells harvested in late exponential phase produce the glucose-lipid surfactant in two forms: a glycine-containing cell-bound precursor that increases the hydrophobicity of the cell and its affinity for oil droplets suspended in the water phase, and a glycine-lacking form that is released from the cell to the medium promoting the formation of oil-water micelles emulsions, and thereby increasing oil bioavailability.

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Alcanivorax hongdengensis, an alkane-degrading bacterium isolated from surface seawater of the straits of Malacca and Singapore, produces a lipopeptide as its biosurfactant (Wu et al. 2009). Strain A–11–3 is capable of utilizing n-alkanes ranging in chain lengths C8–C36. When C16 is the sole carbon source, strain A–11–3 produces a surfactant that contains lipopeptide-like compounds. The major fatty acids in the lipopeptide are C15:0 (46.3%) and C17:0 (40.2%); other fatty acids are present in smaller amounts, including C13:0 (3.3%), C17:1 (6.5%) and C17:2 (3.8%). In the case of the amino acid analysis, two peaks are detected, one of which was identified as tyrosine. The other was not identified.

Schulz et al. (1991) isolated three biosurfactant producing marine bacterial strains among n-alkane degrading microorganisms from marine samples collected around the Isle of Helgoland (North Sea). One strain identified as *Alcaligenes* sp. MM1 produced a glucose lipid, whereas the other two strains belonged to *Arthrobacter* sp. An extracellular emulsifying agent with properties indicating high molecular weight substances were detected from pure cultures of *Arthrobacter* sp. SI 1, whereas trehalose lipids were found to be the biosurfactants produced by *Arthrobacter* sp. EK 1. Chemical analyses and NMR measurements showed that the main fraction was an anionic 2,3,4,2'-trehalose tetraester, containing fatty acids with chain lengths ranging from 8 up to 14 and a succinate moiety linked at C2 atom of trehalose (Passeri et al. 1991).

Pepi et al. (2005) isolated the bacterial strain *Halomonas* ANT–3b from an ice/seawater interface from Terra Nova Bay station, Ross Sea, Antarctica using diesel fuel as sole carbon and energy source. This strain produces a 18 kDa glycolipid emulsifier containing fatty acids and a mixture of sugars (mannose, galactose and glucose).

High molecular weight bacterial surfactants

The high molecular weight bacterial surfactants are high molecular weight polymeric compounds produced by a large number of bacterial species from different genera and are composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers. The high molecular weight surfactants are less effective in reducing interfacial tension, but are efficient in coating the oil droplets and preventing their coalescence. These high molecular weight bioemulsifiers exhibit high substrate specificity. For example, some efficiently emulsify mixtures of aliphatic and aromatic (or cyclic alkane) hydrocarbons, but do not emulsify pure aliphatic, aromatic or cyclic

hydrocarbons; others can also emulsify pure hydrocarbons but only of high molecular weight.

The best-studied biosurfactants are the bioemulsans produced by different species of *Acinetobacter* (Rosenberg and Ron 1998). Among them, the emulsan from *Acinetobacter calcoaceticus* RAG–1, is one of the best characterized. *A. calcoaceticus* RAG-1 (ATCC 31012), a Gram-negative petroleum-degrading marine bacterium isolated from Mediterranean Sea (TelBaruch–Tel Aviv, Israel), can metabolize a variety of carbon sources including crude oils, long chain hydrocarbons, alcohols, fatty acids (FAs), and triglycerides (Reisfeld et al. 1972). During growth, the bacterium secretes an anionic lipoheteropolysaccharide known as emulsan (Rosenberg et al. 1979; Zuckerberg et al. 1979). Emulsan is a high molecular weight heteropolysaccharide containing repeated trisaccharide units of N-acetyl-D-galactosamine, N-acetylgalactosamine uronic acid and an unidentified N-acetyl amino sugar (Zhang et al. 1997). Fatty acids (FA) are covalently linked to the polysaccharide through O-ester linkages (Belsky et al. 1979; Desai and Banat 1997; Zuckerberg et al. 1979). Although emulsan does not reduce interfacial tension as effectively as small molecule surfactants, it is believed that emulsan tightly binds to the surface of oil droplets and thereby prevents coalescence. Saturated and monounsaturated FAs ranging from C10 to C18 are linked to the polysaccharide backbone by O- and N-acyl bonds and constitute between 5 and 23% (w/w) of the polymer (Zhang et al. 1997). The combination of a hydrophilic anionic sugar main chain with hydrophobic FA side chain provides the amphipathic properties to the polymer.

Marine bacterium *A. calcoaceticus* subsp. *anitratus* SM7 was isolated as a bioemulsifier-producing bacterium from oil-spilled seawater in Songkhla lagoon, Thailand. Strain SM7 produced an extracellular high molecular-weight emulsifying agent when grown on *n*-heptadecane as the sole carbon and energy source (Phetrong et al. 2008). Whereas the biosurfactants produced by other *Acinetobacter* strains such as *Acinetobacter calcoaceticus* RAG–1, BD4 and BD413 emulsified efficiently only mixtures of aliphatic and aromatic hydrocarbons (Kaplan and Rosenberg 1982; Rosenberg et al. 1979), the biosurfactant produced by the strain SM7 was able to emulsify pure aliphatic and aromatic hydrocarbons (Phetrong et al. 2008). Furthermore, the stability to pH, temperature and salts make this biosurfactant suitable for environmental applications.

The extremely halotolerant marine bacterium *Marinobacter hydrocarbonoclasticus* (Gauthier et al. 1992), grown on eicosane, produced a high molecular weight extracellular emulsifying agent and adhered to the hydrocarbon, but did not solubilize it before uptake. Both

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emulsification and adherence took place during growth on eicosane, achieving contact between cells and hydrocarbon.

Extracellular glycoprotein bioemulsifiers are also produced by *Halomonas* spp. (Calvo et al. 2002; Martinez-Checa et al. 2002; Pepi et al. 2005). Two marine *Halomonas* species, the strains TG39 and TG67, were selected for their ability to produce high emulsification activities towards hexadecane oil (Gutierrez et al. 2007). Partial purification and chemical-physical characterization of two extracellular water-soluble emulsifying agents produced by the strains TG39 and TG67, named HE39 and HE67 respectively, showed the presence of uronic acid components. Interestingly, as these biopolymers contain high concentrations of charged components, they may also be useful for the bioremediation of toxic metals, as it has been described for other compounds with similar composition (Slizovskiy et al. 2011).

14.3.2 Storage lipids in OHCB

Almost all prokaryotes synthesize lipophilic storage substances when a suitable carbon source is available, whereas nitrogen and/or phosphorus are limiting. The storage lipids serve as energy and carbon sources during starvation periods (Manilla-Perez et al. 2010). Several strains synthesize hydrophobic polymers, such as poly(3-hydroxybutyrate) (PHB) or other types of polyhydroxyalkanoates (PHAs), whereas the accumulation of triacylglycerols (TAGs; trioxoesters of glycerol and long-chain fatty acids) or wax esters (WEs; oxoesters of primary long-chain fatty acids and primary long-chain fatty alcohols) is less common (Wältermann and Steinbüchel 2006).

Crude oil pollution constitutes a temporary condition of carbon excess coupled to a limited availability of nitrogen that prompts marine hydrocarbonoclastic bacteria to accumulate storage compounds. TAGs and WEs production has been frequently reported for species of the genera *Alcanivorax* (Bredemeier et al. 2003; Kalscheuer et al. 2007), *Acinetobacter* (Wältermann and Steinbüchel 2006) and *Marinobacter* (Rontani et al. 1999).

Although most bacterial species store intracellular triacylglycerols and wax esters, in marine organisms the localization of storage lipids is not restricted to the cytoplasm. The production of extracellular wax esters by *Alcanivorax*, *Marinobacter* and *Acinetobacter* strains growing on hexadecane has been described by several authors (Bredemeier et al. 2003; DeWitt et al. 1982; Rontani et al. 1999). The importance of these

compounds as end-products or precursors to produce interesting biotechnologically relevant chemicals has been generally recognized.

The ability of OHCB to metabolize almost exclusively hydrocarbons can be used to produce storage compounds like PHAs, TAGs, and/or WEs as bulk chemicals, while the extracellular deposition of these compounds could represent a major advantage for purification processes. Secretion of lipophilic products into the culture medium rather than its intracellular accumulation can significantly reduce costs of product recovery. Most importantly, microbial production of wax ester has advantages over other biological sources since the wax ester composition can be controlled by carefully choosing starting material or growth conditions.

Alcanivorax jadensis T9, a marine Gram-negative bacterium isolated from the intertidal sediment of the German North Sea coast (Bruns and Berthe-Corti 1999), accumulated TAGs and considerable amounts of extracellular WEs when cultivated on n-alkanes (hexadecane or tetradecane) as carbon source (Manilla-Perez et al. 2010). This strain is considered a suitable candidate for the biotechnological production of extracellular neutral lipids.

Similar features have been reported for *A. borkumensis* SK2 which produce intra- and extracellular (Kalscheuer et al. 2007) TAGs and WEs growing on n-alkanes (hexadecane and octadecane). Although genes coding for enzymes which are required for biosynthesis of bacterial storage compounds like polyhydroxyalkanoates (PHAs), triacylglycerols (TAGs), or wax esters (WEs), are present in the genome (Sabirova et al. 2008; Schneiker et al. 2006) of *A. borkumensis* SK2, only TAGs and WEs seem to be the relevant carbon storage compounds produced by this strain (Kalscheuer et al. 2007). Different carbon sources can drive the synthesis of different storage products. *A. borkumensis* SK2 produces mainly TAGs during cultivation in the presence of pyruvate or acetate, while WEs were synthesized during cultivation on alkanes, such as hexadecane and octadecane (Manilla-Perez et al. 2010). In particular TAG accumulation by *A. borkumensis* SK2 of up to 23% of its cell dry weight has already been reported (Kalscheuer et al. 2007), making this strain a good candidate for large-scale TAG production.

Marinobacter and *Acinetobacter* spp. are known to accumulate wax ester as a storage material. Among them *Marinobacter hydrocarbonoclasticus* SP17 (Klein et al. 2008) and *Acinetobacter* sp. strain M–1 (Ishige et al. 2002) accumulated a large amount of wax esters when grown on n-alkanes under nitrogen limiting conditions. *Marinobacter hydrocarbonoclasticus* DSM 8798 has been reported to

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synthesize isoprenoid wax ester storage compounds when grown on phytol as the sole carbon source under limiting nitrogen and/or phosphorous conditions (Holtzapple and Schmidt-Dannert, 2007).

The formation of isoprenoid wax esters was also reported by Rontani et al. (1999) during aerobic growth of four marine bacteria isolated from hydrocarbon-polluted marine coastal sediments and foams collected from different sites in Gulf of Fos (Mediterranean Sea, France). *Acinetobacter* sp. strain PHY9, *Pseudomonas nautica* (IP85/617), *Marinobacter* sp. strain CAB (DSMZ 11874), and *Marinobacter hydrocarbonoclasticus* (ATCC 49840) produced extracellular isoprenoid wax esters when grown on free phytol and 6,10,14-trimethylpentadecan–2-one, which are two isoprenoid compounds widely distributed in marine sediments (Brassell et al. 1981; Volkman and Maxwell 1986).

Due to the great interest in lipid biotechnology, several enzymes involved in wax esters and TAGs synthesis have been characterized. Low specificity enzymes accepting a broad range of substrate are the most interesting for in vitro biosynthesis of new lipids. Among them, the wax ester synthase/acyl coenzyme A (acyl-CoA):diacylglycerol acyltransferase (WS/DGAT) from *Acinetobacter* sp. strain ADP1 is one of the most characterized (Kalscheuer and Steinbuchel, 2003). This bifunctional enzyme, which mediates both the biosynthesis of wax esters and triacylglycerols, demonstrated an extraordinary low specificity accepting a broad range of saturated and unsaturated fatty alcohols of variable chain length and acyl-CoAs as well as 1,16-hexadecanediol and 1-monopalmitoylglycerol as substrates. The range of possible acyl acceptors for WS/DGAT also includes alkanethiols, such as 1-hexadecanethiol, 1,8-octanedithiol, and 1-*S*-monopalmitoyloctanedithiol, enabling the *in vitro* and also *in vivo* biosynthesis of unusual thio wax esters or dithio wax esters, respectively.

14.4 Enzymes of industrial interest from hydrocarbon-degrading microorganisms

14.4.1 Identification of hydrocarbon-degrading microorganisms

The identification and study of marine microorganisms with unique physiological features can be a powerful tool to discover novel enzymes of possible biotechnological interest (Sanchez-Amat et al. 2010).

Remarkable or unusual bioprocesses are performed by marine biocatalysts due to habitat-related characteristics such as salt tolerance, hyperthermostability, barophilicity and cold adaptivity, which can be desirable characteristics recognized from a general biotechnological perspective (Trincone 2011).

As OHCB are relatively recent discoveries, and have a novel physiology, they might be expected to have enzyme repertoires that are so far unprospected and potentially interesting for the enzymatic biosynthesis of fine chemicals and added value compounds (Yakimov et al. 2007).

In this context a major concern is related to the fact that, although diverse bacteria capable of degrading petroleum hydrocarbons have been isolated and characterized, the vast majority of hydrocarbon-degrading bacteria could remain undiscovered, as a large fraction of bacteria inhabiting marine environments are uncultivable (Eilers et al. 2000). However, the characterization of microbial populations in polluted marine environments without cultivation and the amplification of genes coding for specific enzymes can be carried out by molecular methods.

The increasing number of sequenced genomes of terrestrial and marine oil degrading bacteria available in public databases, and the increasing knowledge about the microbial pathways for hydrocarbon catabolism, actually make it easier to identify marker genes which characterize specific microbial populations. Functional marker genes, encoding key enzymes of characteristic metabolic pathways, are often used to specifically target microorganisms to assign them likely functions in the environment and to isolate enzymes with new biotechnological applications.

In the case of PAH-biodegradation, the most common marker gene used (Habe and Omori 2003) encodes for the large subunit of the catalytic component of the aromatic ring-hydroxylating dioxygenases (ARHDs), which has been shown to confer substrate specificity (Gibson and Parales 2000). Some of these distinct dioxygenase genes were identified in bacteria isolated from the marine environment belonging to the genus *Cycloclasticus* (Geiselbrecht et al. 1998; Kasai et al. 2003), *Nocardioides* (Saito et al. 2000), or *Neptunomonas* and *Pseudoalteromonas* (Hedlund et al. 1999; Hedlund and Staley 2006).

As an example, a well characterized set of genes coding for an ARHD was isolated from *Cycloclasticus* sp. strain A5, able to grow with substituted naphthalenes, dibenzothiophenes, phenanthrenes and fluorenes with or without alkyl substitution. The genes encoded the a (large subunit) and β (small subunit) subunits of an iron–sulfur dioxygenase, a ferredoxin and a ferredoxin reductase, respectively termed

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phnA1, phnA2, phnA3 and phnA4 (Kasai et al. 2003). *Escherichia coli* cells possessing the phnA1A2A3A4 genes were able to convert phenanthrene, naphthalene, methylnaphthalene, dibenzofuran and dibenzothiophene to their hydroxylated forms, thus confirming the hypothetical function based on homology sequence analysis. *AlkB* gene coding for the alkane hydroxylases responsible for the first step of the n-alkanes degradation, is instead the most important marker gene for alkane degrading bacteria (van Beilen and Funhoff, 2007; Vomberg and Klinner 2000; Wang et al. 2010). The biochemical and molecular aspects of *alkB* genes and the enzymes they encode have been relatively well studied, and this has enabled the development of molecular tools for the study of *alkB* genes in the marine environment (Heiss-Blanquet et al. 2005; Kloos et al. 2006; Sei et al. 2003).

14.4.2 Biocatalytic use of oxidative systems from hydrocarbon-degrading marine bacteria

Oxidative systems are not only valuable for the identification of hydrocarbon-degrading microorganisms; these enzymes are becoming a major interest for the biotechnological industry. Reactions in which organic compounds are oxygenated or hydroxylated are in fact of great value for the synthesis of polymers and drugs. However, selective oxyfunctionalization of organic substrates can be a significant problem in organic synthesis, as these reactions are often carried out with strong oxidizing agents and occur with little chemo-, regio-, and enantio-selectivity (Notomista et al. 2011). Therefore, growing attention has been dedicated in recent years to the development of biotransformations which make use of the metabolic versatility of either purified enzymes or whole microorganisms to perform oxyfunctionalization of organic substrates of industrial interest. These methodologies, compared with already established chemical processes, are appealing alternatives to obtain active organic compounds under mild experimental conditions and without employing toxic reagents (Notomista et al. 2011). It is worth noting that, especially for oxidations, the power of enzymes can circumvent the use of halogen-containing and environmentally demanding oxidants, by using oxygen or hydrogen peroxide as clean and selective oxidants (Hollmann et al. 2011).

Hydrocarbon-degrading bacteria are of great significance in this context as biocatalytic oxidation of hydrocarbons such as readily available petrochemicals (e.g., styrene, xylene, octane) and renewable

plant oils (e.g., limonene, valencene) is one of the most valuable transformations for synthetic applications. Chemical counterparts are not competitive because of side reactions and thus have low product yield resulting in high costs for the product recovery (Park, 2007).

Due to their inherent stereo- and regioselectivity and high efficiency, oxidative enzymes have attracted attention as potential biocatalysts for various biotechnological processes. Successful commercial application of these enzymes will be possible through employing new methodologies, such as use of organic solvents in the reaction mixtures, immobilization of either the intact microorganisms or isolated enzyme preparations on various supports, and genetic engineering technology (Sariaslani, 1989). The most representative classes of enzymes that perform this reaction are oxygenases/hydroxylases, peroxidases, and laccases (Di Gennaro et al. 2011). Reactions performed by these enzymes play a significant role in maintaining the global carbon cycle through either transformation or complete mineralization of organic molecules.

So far, there are limited examples of biosynthetic application of oxidative systems isolated from marine bacteria; few oxygenases and laccases have been used for biocatalytic purposes; this consideration should strongly encourage the future activity of enzyme bioprospecting in the marine milieu.

Among the possible substrates for biocatalytic processes, much interest has been recently devoted to the oxidation of polycyclic aromatic compounds, and in particular of naphthalene. It is worth noting that compounds having naphthalene in their molecular structure are candidates for biologically active chemicals, medicines, and commodity chemicals. These novel compounds can be used for further modification such as prenylation, as natural products with one or more prenyl groups have been shown to possess anti-microbial, anti-oxidative, anti-inflammatory and anti-cancer activity (Shindo et al. 2011). Shindo and coworkers reported the bioconversion of 2-methylnaphthalene, 1-methoxynaphtalene, and 1-ethoxynaphthalene to produce 10 novel prenyl naphthalene-ols, by using the combined action of an aromatic dihydroxylating dioxygenase from marine bacterium *Cycloclasticus* sp. strain A5, PhnA1A2A3A4, the prenyltransferase NphB from *Streptomyces* sp. strain CL190, or SCO 7190, a dimethylallyltransferase from *Streptomyces coelicolor*. These novel prenyl naphthalene-ols each showed potent antioxidative activity against a rat brain homogenate model (Shindo et al. 2011).

The bioconversion of various substituted naphthalenes was also the subject of experiments performed by Misawa et al. (2011) which used substrates that contained 1-methoxy- and 1-ethoxy-naphthalenes,

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methylnaphthalenes, dimethylnaphthalenes, and naphthalene carboxylic acid methyl esters. The bioconversion of these substrates was performed using recombinant *E. coli* cells expressing the polycyclic aromatic hydrocarbon (PAH)-dihydroxylating dioxygenase of *Cycloclasticus* sp. strain A5. A variety of novel mono-hydroxylated derivatives were generated from these substituted naphthalenes (Misawa et al. 2011).

Phenanthrene has been another polycyclic aromatic compound used in biotransformation processes. The phdABCD gene cluster of the marine bacterium *Nocardioides* sp. strain KP7, coding for a multicomponent enzyme phenanthrene dioxygenase, was first manipulated and positioned downstream of the thiostrepton-inducible promoter PtipA in a high-copy-number vector pIJ6021, and then introduced into the Gram-positive, soil-inhabiting, filamentous bacterium *Streptomyces lividans* (Chun et al. 2001). The recombinant *S. lividans* cells converted phenanthrene into a cis-diol form, which was determined to be cis–3,4-dihydroxy–3,4-dihydrophenanthrene. Although this example was mainly discussed in terms of bioremediation of PAHs polluted soils by a recombinant *Streptomyces* strain, it should be remembered that cis-diols are considered very useful as building blocks for asymmetric synthesis (Hudlicky et al. 1999). Thus, the phenanthrene dioxygenase of *Nocardioides* sp. strain KP7, which proved to be functional in a heterologous host, is a valid target for the future development of biocatalytic processes based on its enzymatic activity.

The metabolic versatility, hence the biotechnological potential, of this enzyme has also been tested on various tricyclic fused aromatic compounds such as fluorene, dibenzofuran, dibenzothiophene, carbazole, acridene, and phenanthridine. These experiments were done using the cells of *Escherichia coli*. Transformants expressing the phenanthrene dioxygenase (phdABCD) genes derived from the marine bacterium *Nocardioides* sp. strain KP7 converted all of these tricyclic aromatic compounds (Shindo et al. 2001). The cells of a *Streptomyces lividans* transformant carrying the phenanthrene dioxygenase genes were also evaluated for bioconversion of various tricyclic fused aromatic compounds. The ability of this actinomycete in their conversion was similar to that of *E. coli* carrying the corresponding genes. Products converted from the aromatic compounds with these recombinant bacterial cells were purified and identified. Several products, e.g., 4-hydroxyfluorene converted from fluorene, and cis–1,2-dihydroxy–1,2-dihydrophenanthridine, cis–9,10-dihydroxy–9,10-dihydrophenanthridine, and 10-hydroxyphenanthridine, which were converted from phenanthridine, were novel compounds (Shindo et al. 2001).

As previously underlined in this section, laccases are also important enzymatic systems that can be used to perform the oxyfunctionalization of substrates of biotechnological interest. Laccases are blue multicopper oxidases with potential applications in environmental and industrial biotechnology. In a study by Fang and coworkers, a new bacterial laccase gene of 1.32 kb was obtained from a marine microbial metagenome of the South China Sea by using a sequence screening strategy (Fang et al. 2011). The protein (named as Lac15) of 439 amino acids encoded by the gene contains three conserved $Cu^{(2+)}$-binding domains, but shared less than 40% of sequence identities with all of the bacterial multicopper oxidases characterized. Lac15, recombinantly expressed in *E. coli*, showed high activity towards syringaldazine at pH 6.5–9 with an optimum pH of 7.5 and with the highest activity occurring at 45 °C. Lac15 was stable at pH ranging from 5.5 to 9 and at temperatures from 15 to 45 °C. Distinguished from fungal laccases, the activity of Lac15 was enhanced twofold by chloride at concentrations lower than 700 mM, and kept the original level even at 1 000 mM chloride. Furthermore, Lac15 showed the ability to decolorize several industrial dyes of reactive azo class under alkalescent conditions. The properties of alkalescence-dependent activity, high chloride tolerance, and dye decolorization ability make the new laccase Lac15 an alternative for specific industrial applications (Fang et al. 2011).

14.5 References

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